Reaction Mass Formation in *Drosophila*, with Notes on a Phenoloxidase Activation

NOBUHIKO ASADA[1] and TAKASHI FUKUMITSU

Biological Laboratory, Faculty of Science, Okayama University of Science, Okayama 700, Japan

ABSTRACT—In the supernatant fraction of the adult *Drosophila*, phenoloxidase activity was detected by the use of SDS-PAGE and spectrophotometry. The level of phenoloxidase activity increased within six minutes after the initiation of copulation. The length of time of reaction mass formation corresponded to the increase of phenoloxidase activity after the initiation of copulation in a copulated female. The reaction mass formation in mated female *Drosophila* was, in part, considered to be in relation to phenoloxidase activation after copulation.

Abbreviations

Phenoloxidase: *o*-diphenol: O_2 oxidoreductase, EC 1. 10. 3. 1

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

p-NPGB: *p*-nitrophenyl-*p*'-guanidinobenzoate

EDTA: Ethylenediaminetetraacetic acid

Dopa: L-3, 4-dihydroxyphenylalanine

Dopa chrome: 2, 3-dioxyindole-5, 6-quinone-2-carboxylic acid

MW: Molecular weight

K: Kilodalton

INTRODUCTION

In *Drosophila*, reaction mass, which is to be found in the female uterus, is formed immediately after copulation associated with the insemination reaction [1–6]. It takes place just after the initiation of copulation, within 10 min, in both intra- and interspecific crosses. Early studies revealed that it played a role that led to preparation of the reproductive tract for oviposition and had a bearing on the problem of speciation process in intraspecific crosses, because reaction mass remained soft and unmelanized then disappeared,

probably by proteolysis, *in vivo* prior to oviposition. On the other hand, in interspecific crosses, reaction mass that remained in the uterus prevented oviposition resulting in a failure of hybrid production. Therefore reaction mass seems to play an important role not only in fecundity, but also as a primitive self-defense response in *Drosophila*. Biochemical studies revealed that reaction mass formation was likely to be a consequence of polymerization and/or conformational change(s) of phenol-containing substance(s) involving the same course of the melanization cascade reaction [6–7]. In this article, the relationship between the reaction mass formation and the key enzyme for melanization, phenoloxidase, is discussed.

MATERIALS AND METHODS

1. Flies

Wild type flies of *Drosophila nasuta* (ADM-1 strain, Andaman, India; MYS-23 strain, Mysore, India; SEZ-2 strain, Seychelles) and *D. pallidifrons* (PNI-74 and PNI-110 strains, Ponape, Caroline Islands) were used in this study. All flies were iso-female strains caught in nature. *D. pallidifrons* has been regarded as an ancestral species and *D. nasuta* has been thought to be a derived species from *D. pallidifrons* [8]. Culture condition and preparation of flies were performed in the same manner as described in Insemination reaction in the *Drosophila nasuta* subgroup [6].

2. Temperature shift

Two experiments were performed. Virgin females and males, four-day-old, were put together in a glass vial (30 mm in diameter, 110

mm in depth) at 25°C. To observe the copulation, approximately 30 pairs were placed together in the vial. In Experiment-1 (Ex-1), the vials containing the copulating pairs were removed from 25°C and replaced in 2°C within two min after the initiation of copulation. After five hr at 2°C, females were dissected in the *Drosophila* Ringer solution [9] and were examined to determine whether reaction mass was produced in the uterus. In Ex-2, copulating pairs were placed at 2°C in the same manner as Ex-1; then the females were separated to avoid remating, and the culture temperature was raised from 2 to 25°C. After four hr at 25°C, females were dissected and examined. Fifty pairs were run for each experiment.

3. *Preparation of samples*

A crude extract was prepared to test for the presence of phenoloxidase and to study the kinetics of the activity. Five individuals of four-day-old adult flies were homogenized in the solution containing 50 μl of a 0.1 M phosphate buffer solution (PBS), pH 6.0, at 0°C. Homogenate was centrifuged at 12,000 round per min (rpm) for five min at 4°C, then supernatant fraction was subjected to SDS-PAGE. To assay the phenoloxidase activity, adult flies were homogenized in the PBS mentioned above adding 25 μl of a specific serine protease inhibitor, *p*-NPGB dissolved in dimethyl-formamide and diluted with acetonitrile (final concentration 0.01 M) and 25 μl of a 10 mM EDTA.

4. *SDS-PAGE*

The crude extract was dissolved in 50 μl of a sample buffer solution containing 0.0625 M Tris, 2% SDS, 5% 2-mercaptoethanol, pH 6.0, and a small amount of bromothymol blue. Extracts boiled for two min were also prepared. The samples were separated by 10% SDS-PAGE according to the buffer system of Laemmli [10] for the slab gel (2 mm gel thickness). Electrophoresis was run for about 10 hr at a constant current of 20 mA, 65 V at the initial stage. Phenoloxidase activity was detected by staining with a 0.013 M dopa in a 0.1 M PBS, pH 6.0 used as a substrate buffer solution for several min at 37°C.

5. In vitro *melanization and phenoloxidase activity*

In vitro melanogenesis of reaction mass was performed. A single reaction mass collected from the uterus of a copulated female was rinsed three times in a PBS, pH 6.8, and then soaked at 25°C in a substrate of 5×10^{-5} M dopa. Melanization of the reaction mass was observed at regular intervals, and the score was expressed in the same manner as Hiruma and Riddiford [11].

Phenoloxidase activity was determined at 2, 5, 10 and 15 min from the beginning of the initiation of copulation. The reason for these specific times being that the average duration of copulation in intraspecific crosses of *D. nasuta* and *D. pallidifrons* and interspecific crosses between *D. pallidifrons* females and *D. nasuta* males was approximately 16, 10 and 12 min, respectively [6]. Phenoloxidase activity was assayed from the supernatant fraction just after adding 1 ml of a PBS and 1 ml of a 30% acetic acid. The color intensity of dopa chrome was measured colorimetrically by a Hitachi Model-101 Spectrophotometer at 475 nm. Two replications were run at each observation time.

RESULTS AND DISCUSSION

Temperature dependency and in vitro *melanization of reaction mass*

Results of Ex-1 and Ex-2 are summarized in Table 1. In the controled experiment, high frequencies of reaction mass formation was obtained in intraspecific crosses of *D. nasuta* and there were no significant differences in the t-test among the three strains. The frequency being so low, approximately 20.0% in the intraspecific crosses of *D. pallidifrons*, no further analysis was performed.

In Ex-1, the difference in the frequency of the reaction mass formation was highly significant in all crosses as compared with the control group. Reduction ranged from 60.0%, ADM-strain, to 33.0%, SEZ-strain, in the intraspecific crosses of *D. nasuta* as compared to the corresponding control figures of 86.0–90.0%. No complete

TABLE 1. Percent of reaction mass formation in temperature shift experiments

Cross type				% of formation of reaction mass		
Species	Strain		Number of females	Control	Ex-1	Ex-2
	Female	Male				
Drosophila nasuta	ADM-1	ADM-1	50	90.0	36.0	70.0
×	MYS-23	MYS-23	50	90.0	52.0	70.0
D. nasuta	SEZ-2	SEZ-2	50	86.0	58.0	68.0
D. pallidifrons	PNI-74	PNI-74	50	12.0	nd	nd
×						
D. pallidifrons	PNI-110	PNI-110	50	20.0	nd	nd
D. pallidifrons	PNI-74	SEZ-2	50	52.0	24.0	46.0
×						
D. nasuta	PNI-110	SEZ-2	50	94.0	46.0	82.0

(Significance markers: ADM-1: Control–Ex-1 ***, Ex-1–Ex-2 ***; MYS-23: Control–Ex-1 ***, Ex-1–Ex-2 ns; SEZ-2: Control–Ex-1 **, Ex-1–Ex-2 ns; PNI-74×SEZ-2: Control–Ex-1 **, Ex-1–Ex-2 *; PNI-110×SEZ-2: Control–Ex-1 ***, Ex-1–Ex-2 ***)

* p<0.05, ** p<0.01, *** p<0.001.
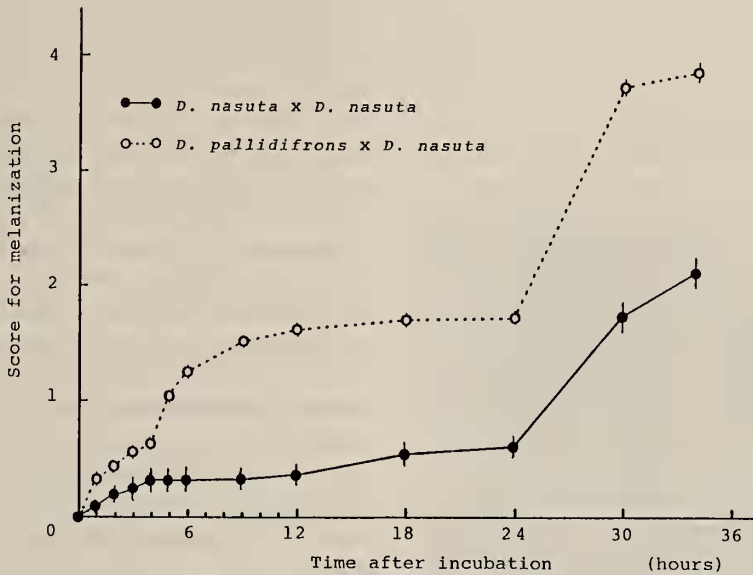ns: not significant, nd: not done.

FIG. 1. Melanization of reaction mass incubated with dopa *in vitro*. Score of melanization is expressed after Hiruma and Riddiford (1984).

inhibition, however, was observed at the low temperature. As shown in Ex-2, recovery of the reaction mass formation observed in all crosses was especially significant in intraspecific crosses of *D. nasuta*, ADM-1 strain, and interspecific crosses between *D. pallidifrons* females, PNI-110 strain, and *D. nasuta* males, SEZ-2, strain when the copulated females were re-transferred from 2 to 25°C. The frequencies, however, were lower than the control values for all crosses.

In vitro melanogenesis was confirmed after incubation with dopa as shown in Figure 1. Length

of time necessary for melanization was much longer in intraspecific than in interspecific crosses. The larger the score increase, the darker the color of the reaction mass. Length of time of melanization reflects that *in vivo*; the reaction mass is somewhat rigid, then colored dark only in interspecific crosses as shown in the previous study [6]. The present work demonstrates that stimulation by copulation itself of some sexual substances ejaculated by the male play an important role in activation of phenoloxidase *in vivo* as the trigger in a copulated female. Additionally, phenoloxidase plays a key role in the formation of reaction mass. Firstly, because melanization of the reaction mass occurred both *in vivo* [6] and *in vitro* (Fig. 1) especially in interspecific crosses. Secondly, formation of reaction mass was significantly inhibited by a direct injection of phenoloxidase inhibitors, thiourea and sodiumdiethyldithiocarbamate [7].

Detection of phenoloxidase by SDS-PAGE and spectrophotometry

Phenoloxidase activity in the extracts from adult flies were examined by SDS-PAGE. As presented in Figure 2, the arrow indicates active bands for

FIG. 2.   SDS-polyacrylamide gel electrophoresis of phenoloxidase in adult fly.

Lane-1: marker protein, 2: marker protein, 3: *D. nasuta* (SEZ-2 strain), non-heated, 4: *D. nasuta* (SEZ-2 strain), heated, 5: *D. pallidifrons* (PNI-74 strain), non-heated, 6: *D. pallidifrons* (PNI-74 strain), heated, 7: *D. pallidifrons* (PNI-110 strain), non-heated, 8: *D. pallidifrons* (PNI-110 strain), heated.

phenoloxidase in lanes-3, 5, and 7 appeared within six minutes and then darkened during incubation with dopa at 37°C.   Three active bands were distinguishable and molecular weights of each protein were estimated with marker proteins; the major protein was 323,000 daltons, the minor proteins were 339,000, and 302,000 daltons (data not shown). In comparison with active bands in *D. nasuta* and two strains of *D. pallidifrons,* banding patterns were generally similar to one another. No active bands, however, could be detected when the extracts were heated at 100°C as shown in lanes 4, 6, and 8.

Insect phenoloxidase exists as a proenzyme that is processed to become active by protein denaturation and proteases [13]. In *Drosophila*, there are some proenzymes including three A components activated by a natural activator isolated from pupae [14] and one P activating component by 2-propanol [15]. In *D. melanogaster*, three phenoloxidases have been found; one with a monophenoloxidase activity (tyrosinase) and two with dopa oxidase activities [16–17]. The active bands in the gels in this study seems to be dopa oxidase rather than tyrosinase because the extracts obtained from adults were incubated not in tyrosine but in dopa, resulting in the three active bands as given in Figure 2.

Concerning the previous study in *Drosophila*, phenoloxidase activity could be detected in the first-instar-larva, and began to rise, then reached a maximum level at the time of puparium formation. Activity decreased at the stage of pupa, then recovered after eclosion [18–21]. Electropherogram of the present study showed the developmental profile of phenoloxidase activity, that is, the highest activity of phenoloxidase was detected at the late third-instar-larva and not at pupa (data not shown).

Phenoloxidase activity in a supernatant fraction from the whole body of female flies is given in Figure 3. A virgin female was used as the control. The highest level of phenoloxidase activity was presented at five minutes after the initiation of copulation, and no significant differences were observed in all cross types. Then the phenoloxidase activity decreased gradually.   The data suggests that the positive correlation between the
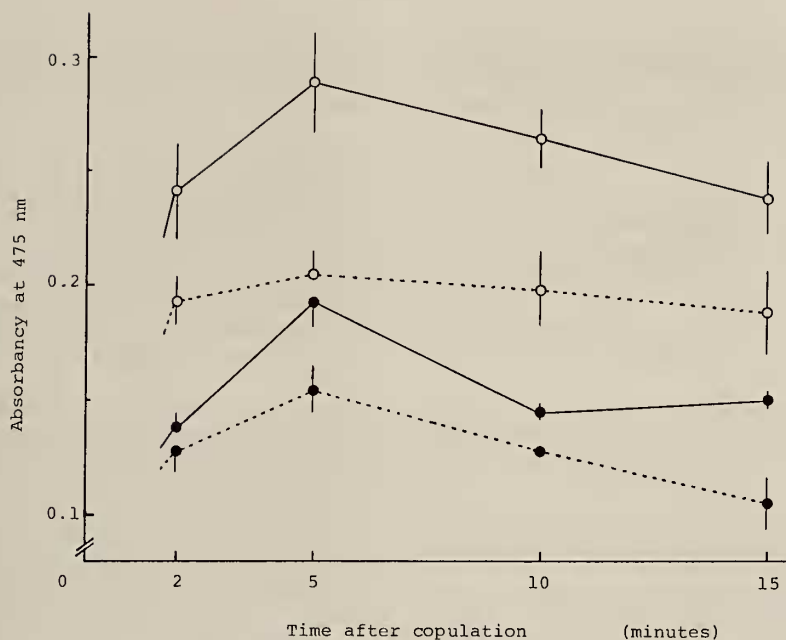
FIG. 3.   Phenoloxidase activity in copulated female *in vitro*.

●———● *D. nasuta* × *D. nasuta*
●------● *D. nasuta* (♀, control)
○———○ *D. pallidifrons* × *D. nasuta*
○------○ *D. pallidifrons* (♀, control)

formation of reaction mass in the uterus and phenoloxidase activation in the supernatant fraction, and that phenoloxidase seems to make a certain contribution to reaction mass formation. The reaction mass formation was temperature dependent; in other words, the frequency was significantly reduced at a low temperature ($2°C$), but it recovered when the copulated females were placed again under suitable conditions (Table 1). However, no phenoloxidase activity appeared when the extracts heated at $100°C$ showed a lack of mass formation. The length of time of phenoloxidase activity was quite similar to that of the reaction mass formation (Fig. 3).

The phenoloxidase system in insects is quite important not only in melanin synthesis and the formation of the cuticle, but also in primitive defense responses in *Drosophila* [7]. In *D. melanogaster,* some mutants which are known to influence melanogenesis, for example, the relationship between *lozenge* (*lz*) and various components of phenoloxidase complex; that is, the A_1 activity is absent in the mutant *lozenge-grossy*

(lz^g), the A_2 activity is reduced in *lz* [20] and some electrophoretic variants of phenoloxidase in *lz* mutant have been identified [17]. The phenoloxidase system, however, might be related to reaction mass formation in a manner that may play an important role in preventing gene exchange and interspecific hybridization. Molecular analysis of the phenoloxidase system is needed for further understanding of this system.

REFERENCES

1   Patterson, J. T. (1946) A new type of isolation mechanism in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A., **32**: 202–208.

2 Patterson, J. T. (1947) The insemination reaction and its bearing on the problem of speciation in the *mulleri* subgroup. Univ. Texas Publ., **4720**: 42–77.

3 Wheeler, M. R. (1947) The insemination reaction in the intraspecific matings of *Drosophila*. Univ. Texas Publ., **4720**: 78–115.

4 Patterson, J. T. and Stone, W. S. (1952) Evolution in the Genus Drosophila, Macmillan, New York.

5 Takanashi, E. (1983) Genetic and reproductive differentiation in *Drosophila sulfurigaster*. Jpn. J. Genet., **58**: 463–485.

6 Asada, N. and Kitagawa, O. (1988) Insemination reaction in the *Drosophila nasuta* subgroup. Jpn. J. Genet., **63**: 137–148.

7 Asada, N. and Kitagawa, O. (1988) Formation and the inhibition of reaction plug in mated *Drosophila*-Study of a primitive defense reaction. Dev. Comp. Immunol., **12**: 521–529.

8 Asada, N. and Kitagawa, O. (1982) Reproductive isolating mechanisms in the *D. nasuta* subgroup I. Jpn. J. Genet., **57**: 658–659.

9 Ephrussi, B. and Beadle, G. W. (1936) A technique of transplantation for *Drosophila*. Amer. Natl., **70**: 218–225.

10 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

11 Hiruma, K. and Riddiford, L. M. (1984) Regulation of melanization of tobacco hornworm larval cuticle *in vitro*. J. Exp. Zool., **230**: 393–403.

12 Ashida, M. (1971) Purification and characterization of prephenoloxidase from hemolymph of the silkworm *Bombyx mori*. Arch. Biochem. Biophys., **144**: 749–762.

13 Ashida, M. and Dohke, K. (1980) Activation of pro-phenoloxidase by the activating enzyme of the silkworm, *Bombyx mori*. Insect Biochem., **10**: 37–47.

14 Mitchell, H. K. and Weber, U. M. (1965) *Drosophila* phenol oxidase. Science, **148**: 964–965.

15 Batterham, P. and McKechnie, S. W. (1980) A phenol oxidase polymorphism in *Drosophila melanogaster*. Genetika, **54**: 121–126.

16 Rizki, T. M. and Rizki, R. M. (1984) The cellular defense system of *Drosophila melanogaster*. In "Insect Ultrastructure". Ed. by R. C. King, and H. Akai, Plenum Publishing Co., New York, pp. 579–604.

17 Rizki, T. M., Rizki, R. M. and Bellotti, R. A. (1985) Genetics of *Drosophila* phenoloxidase. Mol. Gen. Genet., **201**: 7–13.

18 Ohnishi, E. (1953) Tyrosinase activity during puparium formation in *Drosophila melanogaster*. Jpn. J. Zool., **11**: 69–74.

19 Geiger, H. R. and Mitchell, H. K. (1966) Salivary gland function in phenol oxidase production in *Drosophila melanogaster*. J. Insect Physiol., **12**: 747–754.

20 Mitchell, H. K. (1966) Phenol oxidase and *Drosophila* development. J. Insect Physiol., **12**: 755–765.

21 Peeples, E., Geisler, A., Whitcraft, C. J. and Oliber, C. P. (1969) Activity of phenol oxidase at the puparium formation stage in development of nineteen *lozenge* mutant of *Drosophila* melanogaster. Biochem. Genet., **3**: 563–569.